

5 **We Claim:**

1. A method for detecting pathogenic mycobacteria in clinical specimens said method comprising steps of:

- (a) clarifying the clinical specimens from contaminant including mucus by conventional methods,
- 10 (b) treating the processed clinical specimens obtained in step (a) with the modified lysis buffer to inactivate the live pathogenic mycobacteria to make the process safe for the user,
- 15 (c) extracting genomic DNA from the processed clinical specimen obtained from step (b) using a modified method to increase the yield and quality of DNA,
- 20 (d) designing sequence of SEQ ID No. 4 from the DNA obtained in step (c) for specific detection of pathogenic mycobacteria, said designed sequence comprising of selected intergenic region of SEQ ID No. 3, a flanking region containing a portion of the gene mmaA1 of SEQ ID No. 1 and a portion of gene mmaA2 of SEQ ID No.2 of the DNA obtained in step (c),
- 25 (e) designing and synthesizing a set of specific oligonucleotide primers of SEQ ID No. 5, which is the forward primer and SEQ ID No. 6, which is the reverse primer for Polymerase Chain Reaction (PCR) amplification of SEQ ID No. 4,
- 30 (f) developing a PCR amplification process for specific amplification of SEQ ID No.4 of (d) said process comprising using the specific oligonucleotide primers of designed and synthesized in step (e) for detecting presence of pathogenic mycobacteria in the clinical specimens and
- (g) analysing the amplified PCR product by restriction fragment length polymorphism (RFLP) analysis for differentiation of the species of

5 the pathogenic mycobacterium for a quick assessment of HIV co-infection.

2. A method as claimed in claim 1, wherein the designed SEQ ID No.4 is having a sequence as follows:

10 5'GAGGTGTAATGCCTTTCCGGACCCTAGGTGGCCTTTCGGTGCTTGACAG
GAACGCACCGATGCTTCCCCCTCCCCGCATGCTCGAGGCATGCTATCCGA
TACAGGGCCGCCGCACTAAACCGCGATCGAATTTGCCCAGGTCAGGGAA
CGGATATGAGCGGACGAG 3'

- 15 3. A method as claimed in claim 1, wherein, the clinical specimen is selected from sputum, gastric lavage, cerebrospinal fluid, blood, tissue biopsies, or bone marrow aspirates and other body fluids or tissues.

- 20 4. A method as claimed in claim 1, wherein, clarification of specimen in the step (a) from the contaminants (live organisms other than mycobacteria and mucus) is carried out by digestion decontamination mix containing mild alkali, NaOH, tri sodium citrate and a mucolytic agent and guanidinium isothiocyanate in the range of about 0.4-2.5 M followed by concentrating the specimen by centrifugation.

5. A method as claimed in claim 4, wherein, the digestion decontamination mix containing mild alkali, NaOH, tri sodium citrate and a mucolytic agent and guanidinium isothiocyanate in the range of about 0.5-2.0 M.

- 25 6. A method claimed in claim 1, wherein, the DNA in the step (c) is extracted from the treated clinical specimen using a modified lysis buffer by inclusion of ingredients comprising guanidinium isothiocyanate in range of about of 0.5-8 M, Tris.Cl pH 7.6 is in the range of about 20-100 mM, N lauryl Sarcosyl is in range of about 0.5- 2 % of, EDTA is in the range of about 0.1-20 mM, β -Mercaptoethanol is in the range of about 1-25 mM and NH_4COOH is in the range of about 0.3M-1M and purifying the
30 DNA to improve yield by thorough precipitation by organic solvents.

- 5 7. A method claimed in claim 6, wherein, guanidinium isothiocyanate is about 4 M, Tris.Cl pH 7.6 is about 50 mM, N lauryl Sarcosyl is about 1 % of, EDTA is about 1 mM, β -Mercaptoethanol is about 10 mM and NH_4COOH is about 0.7M.
8. A method as claimed in claim 6, wherein, the organic solvents are selected from group comprising of phenol/chloroform mixture and chloroform
- 10 9. A method claimed in claims 1 and 6, wherein, the genomic DNA yield is increased in the range of about 25 to 50 %.
10. A method claimed in claim 9, wherein, the genomic DNA yield is increased in the range of about 30 to 40 %.
- 15 11. A method as claimed in claim 1, wherein, high yielding amplification of DNA in step (f) is achieved by the modified Touch down PCR cycling conditions said conditions comprising steps of initial high annealing temperature in the range of about 62-72 °C followed by lowering of temperature in the range of about 0.2 - 1 °C per PCR cycle for the first 10-25 cycles, which is the touch down step to an optimum annealing temperature of about 56-62 °C for another 30 PCR cycles.
- 20 12. A method as claimed in claim 13, wherein, high yield amplification of DNA is achieved by modified Touch Down PCR cycling conditions, said conditions comprising steps of initial high annealing temperature of about 70°C followed by lowering temperature of about 0.8 °C per PCR cycle for about first 14 cycles to about 58 °C for another 25 PCR cycles.
- 25 13. A method as claimed in claim 1, wherein, the oligonucleotide primers capable of amplification of intergenic region of SEQ ID No. 4 for detection of pathogenic Mycobacteria in clinical specimens are selected from group:
- a. 5' TGGATCCGTTGACCATGAGGTGTAATG 3'(SEQ ID No. 5), which is the forward primer.
- 30 b. 5' GGAATTCCACTACCACGGACTCTC 3' (SEQ ID No. 6), which is the reverse primer.
14. A method as claimed in claim 1, wherein, the length of oligonucleotide primers is between 5 and 100 bases.

- 5 15. A method as claimed in claims 1, wherein, the modified lysis buffer provides a cleaner preparation of the DNA.
16. A method as claimed in claim 1, wherein, treatment with the modified lysis buffer containing 4M guanidinium isothiocyanate inactivates the live mycobacteria to make the procedure safer for the operator
- 10 17. A diagnostic kit for the detection of pathogenic mycobacteria in clinical specimens comprising primers selected from the group consisting of:
- a. 5' TGGATCCGTTGACCATGAGGTGTAATG 3' (SEQ ID No. 5), which is forward primer, and
- b. 5' GGAATTCCACTACGCACGGACTCTC 3' (SEQ ID No. 6), which is the reverse primer
- 15